Br. J. Pharmac. (1979), **65**, 7–13

MORPHINE DEPRESSION AND TOLERANCE OF NERVE-INDUCED PAROTID SECRETION

S.R. BOWEN & F.G. CARPENTER

Department of Pharmacology, University of Alabama Medical Center, Birmingham, Alabama 35294, U.S.A.

1 The nerve-induced secretion produced by the rat parotid gland is proportional to the frequency of stimulation. Morphine decreased the flow rate during stimulation at 2.5 and 5 Hz, but not at 20 Hz. This frequency-dependent action of morphine was partially reversed by naloxone.

2 The secretion produced by the rat parotid gland during an intravenous infusion of acetylcholine was not diminished by morphine. Therefore, the action of morphine on nerve-induced secretion is most probably on the motor nerve terminals, which release acetylcholine.

3 Animals that had been implanted with morphine base pellets tolerated 4 times as much morphine as controls; after 6 days the minute ventilation was less depressed by graded doses of morphine than non-implanted controls.

4 Nerve-induced secretion in morphine-implanted animals was less depressed by morphine than control animals 6 and 24 h after the pellets were removed. The flow rates in the 6 h group treated with morphine were greater after naloxone than control (precipitated withdrawal) but at 24 h when withdrawal symptoms were no longer evident, naloxone produced only a slight reversal.

Introduction

Morphine inhibits the release of acetylcholine (ACh) in the brain *in vivo* (Beleslin & Polak, 1965; Jhamandas, Phillis & Pinsky, 1971) and from brain slices *in vitro* (Sharkawi & Shulman, 1969) in the same relatively low concentrations which produce euphoria, analgesia and respiratory depression. At these same concentrations, morphine also reduces contractions of the electrically stimulated guinea-pig ileum (Paton, 1957) and mouse vas deferens (Henderson, Hughes & Kosterlitz, 1974). In this study, the effects of morphine on the peripheral cholinergic junction of the rat parotid gland were examined *in vivo*. The use of a secretory autonomic effector extended previous studies performed on various smooth muscle preparations and provided further insight into the neuronal mechanisms of opiate dependence.

The parotid gland offers several advantages for a study of the action of morphine on junctional transmission. In the anaesthetized rat, parotid secretion can be readily elicited by stimulation of its parasympathetic postganglionic motor nerves (Schneyer & Hall, 1963; Woodruff & Carpenter, 1973). These nerves, which are exclusively cholinergic, may be exposed as a distinct trunk by appropriate dissection and mounted on small electrodes for purposes of electrical stimulation. The volume of saliva that is produced during stimulation is related hyperbolically to the stimulus frequency in the range 2.5 to 20 Hz (Woodruff & Carpenter, 1973). The flow rate at these frequencies is remarkably consistent and at low stimulus rates the volume produced during a given interval can be measured very accurately. The latter is especially important since the action of morphine is usually observed only at low stimulus rates (Cairnie, Kosterlitz & Taylor, 1961).

In our study, the effects of morphine on nerve-induced parotid secretion were observed in untreated rats and in rats made tolerant to morphine after the implantation of specially formulated morphine pellets (Gibson & Tingstad, 1970). The extent of the tolerance which subsequently developed following implantation was measured directly; the pulmonary minute ventilation of anaesthetized animals was observed before and after the administration of graded doses of morphine.

Methods

Male Sprague-Dawley rats (350 to 450 g) were used in all experiments; they were maintained on food and water *ad libitum* until the time of the experiment. Rats were anaesthetized with Dial-urethane, 0.7 ml/kg (70 mg/kg of diallyl-barbituric acid and 280 mg/kg ethyl carbamate) intraperitoneally. A cannula inserted into the trachea allowed frequent aspiration of any

0007-1188/79/010007-07 $01.00

secretions which accumulated during the procedures. Body temperature was maintained at 37°C with a warming board. Morphine and naloxone were administered into the femoral vein via a 27 G needle by rapid injection.

Parotid gland

After retraction of the lacrimal gland, the auriculotemporal nerve was located beneath the masseter muscle. This muscle was separated from the temporalis muscle by blunt dissection. The nerve could usually be traced proximal to the foramen ovale in the vicinity of the zygoma and the condyloid process of the mandible. This nerve contains postganglionic cholinergic fibres which originate from cell bodies in the otic ganglion. Square wave pulses (2.5, 5 and 20 Hz) were applied through small Pt electrodes placed under the nerve and immersed in mineral oil. The stimuli were of constant duration (1 ms) and varied between 6 to 9 V to provide a supramaximal stimulus.

A stainless steel cup was cemented over the orifice of Stenson's duct with a tissue adhesive (Woodruff & Carpenter, 1973). The cup itself was connected to a graduated pipette with Tygon tubing. Salivary flow rates were determined by measurement of the amount of saliva that had accumulated in the micropipette at 1 min intervals throughout a 5 min collection period. Each animal served as its own control; salivary flow was always determined in each animal before any drugs were given. In control animals, the flow rate remained relatively constant over a period of several hours.

Since doses of morphine were administered which produced hypoventilation it was necessary to maintain ventilation in the animals with a positive pressure respirator.

Acetylcholine infusion

Parotid secretion was also induced by infusing a solution of ACh (10^{-2} M) into the left external jugular vein through a small polyethylene cannula at a rate of 5×10^{-7} mol/min. This solution, administered at a rate of 50 µl/min, resulted in a total volume of no more than 1.0 ml being given to each animal. Each animal also served as its own control. As soon as secretion began, the salivary flow was measured at 1 min intervals during a 5 min infusion period. Coincident with the secretion elicited during the infusion, other autonomic effects were also observed, including pupillary constriction, bradycardia and bloody tears.

Ventilation

Pulmonary minute volume was measured in anaesthetized animals by collection of the expired air in a 2.5 l spirometer over a 10 min period (De Haven & Carpenter, 1964). The displacement of the cylinder was recorded at 1 min intervals. Ventilation was measured only when the animals body temperature had stabilized at 37°C and while the animal was breathing room air. In Figure 4, pulmonary minute volume is expressed at BTPS. Control measurements were made on each animal repeatedly until a uniform minute ventilation rate was established. With this method, ventilation over a 10 min period was always found to be linear and consistent from one animal to another. This suggested that both the spirometer and valve system did not place an undue burden on the animal's ventilatory system. In untreated anaesthetized rats (about 400 g) the mean rate of ventilation amounted to 145 ± 4 ml/min. The empirical formula of Guyton (1947) provided a figure of 156 ml/min for animals of this body weight.

Pellet implantation

Each pellet contained 75 mg of morphine base together with various binding agents (Gibson & Tingstad, 1970). The animals were anaesthetized with diethyl ether and 2 pellets were implanted subcutaneously in the dorsum; 24 h later, 2 additional pellets were implanted. All 4 pellets were removed either 6 or 24 h before the experiment was begun.

Naloxone (40 mg/kg) when given to tolerant animals produces a number of somatic effects which, surprisingly, are manifested in an anaesthetized restrained animal. Of immediate concern was the apparent arousal of the animal as revealed by the production of 'shakes' when the injection was made. With the stimulating electrodes attached to the auriculotemporal nerve of an implanted animal so treated, the nerve itself frequently became injured during these movements. To reduce the intensity of this 'precipitated withdrawal' it was necessary to remove the pellets at least 6 h before conducting an experiment, especially when nerve-induced secretion was measured. Naloxone when given in the same amount to anaesthetized untreated animals produced no significant effect on nerve-induced salivation or on the pulmonary minute volume.

Cation concentration of parotid saliva

Saliva produced during stimulation of the auriculotemporal nerve at 2.5 Hz was collected and analyzed for Na^+, K^+ and Ca^{2+} with a Perkin Elmer atomic absorption spectrophotometer. In both control and

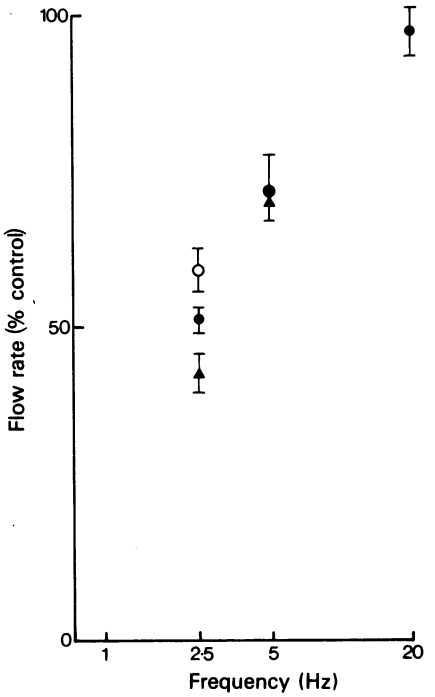

Figure 1 The effect of morphine 1 mg/kg (○), 5 mg/kg (●) and 25 mg/kg (▲) on nerve-induced secretion by the rat parotid gland during stimulation at 2.5, 5 and 20 Hz (abscissa scale). The mean flow rate is expressed on the ordinate scale as percentage of control; vertical lines show s.e. means. $n = 12$.

morphine-treated (25 mg/kg) animals, equal volumes of saliva were obtained before dilution.

Drugs

Acetylcholine chloride (Sigma), diallylbarbituric acid-urethane (CIBA), morphine sulphate (Merck, Sharpe and Dohme) and naloxone hydrochloride (Endo) were used in addition to morphine base pellets. The doses of morphine refer to the salt. Each pellet contained 75 mg of morphine base (Gibson & Tingstad, 1970).

Results

Depression of nerve-induced salivation by morphine

Repetitive stimulation of the auriculotemporal nerve produces a continuous flow of saliva from the parotid gland which is related to the stimulus frequency in the range 2.5 to 20 Hz (Woodruff & Carpenter, 1973). At 20 Hz a mean of 52 ± 3 µl/min of saliva was produced during 5 min of continuous stimulation. In contrast, only 9.6 ± 1 µl/min of saliva was secreted by the gland during stimulation at 2.5 Hz and, at 5 Hz, the flow rate amounted to 19 ± 1 µl/min. Morphine depressed the secretory flow rate at the lowest stimulation frequency (2.5 Hz) but not at 20 Hz. It was also found that the depression of secretion at 2.5 Hz was dose-related. In Figure 1, salivary flow rate was reduced to 50% of control values at the lowest frequency (2.5 Hz). The three mean values expressed in Figure 1 were obtained following treatment with 1, 5 or 25 mg/kg morphine. With an analysis of variance, the depression at 2.5 Hz by 1 mg/kg was found to be no different from 5 mg/kg. However, the 1 mg/kg and 5 mg/kg were each different from the 25 mg/kg; at 5 Hz no significant difference could be shown between the doses.

The major determinant of blockade by morphine was, thus, the stimulation frequency and the dose administered; junctional transmission was impaired during low frequency activity of the nerve terminals. At 5 Hz the interference by morphine was less marked than at 2.5 while, at 20 Hz, the reduction observed after a 5 mg dose of morphine was not different from control (Figure 1).

Naloxone reversal of morphine depression

Naloxone, even in relatively high doses, is a highly specific antagonist of morphine action and is superior to partial agonist compounds with strong antagonist action, such as nalorphine (Blumberg, Dayton & Wolf, 1966). The depression produced by morphine on secretory flow rate was partially reversed by naloxone (40 mg/kg) (Figure 2). The depression of secretory flow elicited at 2.5 and 5 Hz by 25 mg/kg morphine is shown by the height of the open columns in Figure 2. The results are expressed as percentages of the control flow rates obtained before the administration of morphine. The extent to which naloxone was able to reverse the depression caused by this dose of morphine is shown by the height of the hatched column. A paired t test ($P < 0.05$) showed that the salivary flow rates at 2.5 Hz were significantly different following naloxone.

Acetylcholine infusion

In order to test the hypothesis that morphine might exert a depressant action on the postjunctional cholinoceptors of the parotid gland, salivary secretion was induced by the continuous infusion of ACh at a rate of 5×10^{-7} mol/min. Figure 3 shows the mean cumulative volumes which were produced at 1 min intervals during the infusion of ACh. The secretory rate in control animals resulting from the infusion

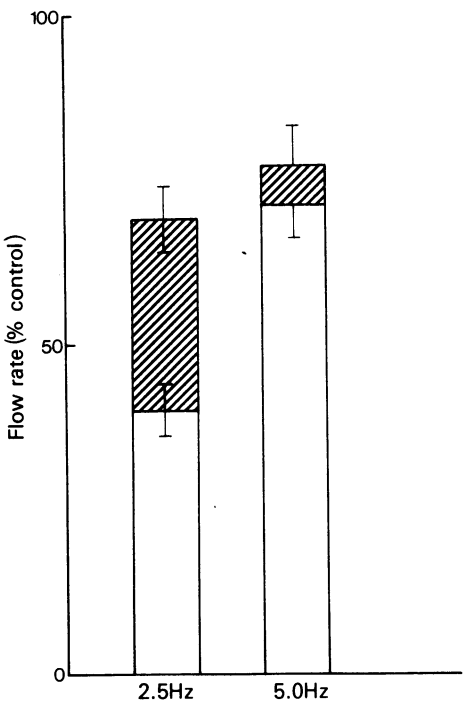

Figure 2 The effect of morphine 25 mg/kg before (open columns) and after naloxone 40 mg/kg (hatched columns) on nerve-induced secretion by the rat parotid gland during stimulation at 2.5 and 5 Hz. The mean flow rate is expressed on the ordinate scale as percentage of control. Vertical lines show s.e. means.

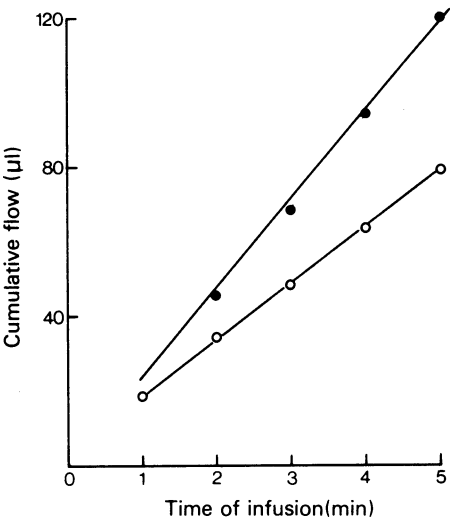

Figure 3 The secretion by the parotid gland elicited during the continuous intravenous infusion of acetylcholine (5×10^{-7} mol/min). Each point is the mean of 6 animals before (○) and after (●) the administration of 5 mg/kg morphine. The volume produced during the infusion is shown on the ordinate and the duration of infusion (min) is shown on the abscissa scale.

amounted to 16 µl/min which was approximately the same as that produced by the gland during electrical stimulation of the auriculotemporal nerve at 5 Hz. After the animals were treated with morphine (5 mg/kg) the flow rate increased significantly to 24 µl/min ($P < 0.05$).

When the data from these experiments were subjected to a linear regression analysis both control and treated lines in Figure 3 gave correlation coefficients of 0.99 or greater; salivary flow rate shown by the slope of each line was constant throughout the 5 min infusion period. As shown in Figure 3, the flow rate actually increased in each animal following morphine treatment. Moreover, in a number of pellet-implanted animals, the volume of saliva secreted during ACh infusion after morphine (5 mg/kg) was found to be identical to that of non-implanted animals. Accordingly, a postjunctional depression of the secretory cells of the parotid gland by morphine seems unlikely.

The development of morphine tolerance in adult rats

The pulmonary minute volume was measured in anaesthetized adult male rats maintained at a constant temperature of 37°C. Over a 10 min period the mean expired volume measured in 19 animals was 145 ± 4 ml/min. This figure is in agreement with previous reports by Guyton (1947), Masland & Yamamoto (1962) and Bartlett & Tenney (1970). The resting pulmonary minute volume of the control or naive rats was depressed substantially by morphine (1 to 2 mg/kg). As shown in Figure 4, the mean depression of pulmonary minute volume was 19% after 1 mg/kg of morphine and 32% after the administration of 2 mg/kg. A dose of 5 mg/kg administered to control animals resulted in ventilatory arrest or apnoea in 7 out of 16 animals. An analysis of variance showed the pulmonary minute volumes were significantly different for each dose. However, morphine did not depress pulmonary minute volume to the same extent in the rats which had had morphine pellets implanted for 6 days. In the control animals, 5 mg/kg morphine resulted in 63% depression of the pulmonary minute volume, which was depressed by only 34% in morphine-pretreated rats given this dose. Moreover, the implanted animals tolerated doses of morphine as high as 20 mg/kg (Figure 4).

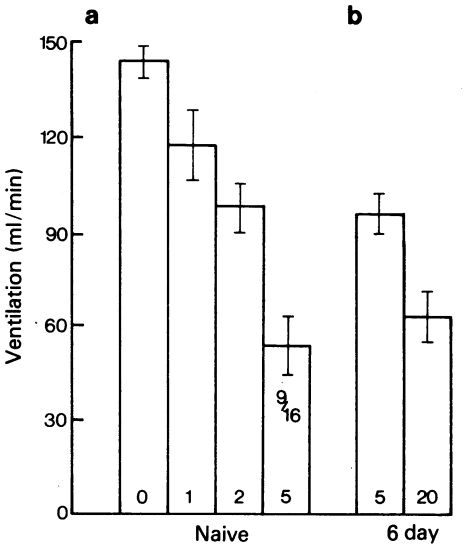

Figure 4 The effect of increasing doses of morphine on the pulmonary minute volume of control and morphine-pretreated rats. In (a) the columns indicate the mean ventilation in 19 control animals before (0) and after 1, 2, and 5 mg/kg i.v. morphine; 7 out of 16 animals showed ventilatory arrest or apnoea following the highest dose and were not included in the result. In (b) the columns indicate the mean pulmonary minute volume of pellet-implanted animals (6 days) after 5 and 20 mg/kg morphine. Vertical lines show s.e. means in (a) and (b). Ordinate scale: pulmonary minute volume (ml/min); the dose of morphine (mg/kg) is shown within each column.

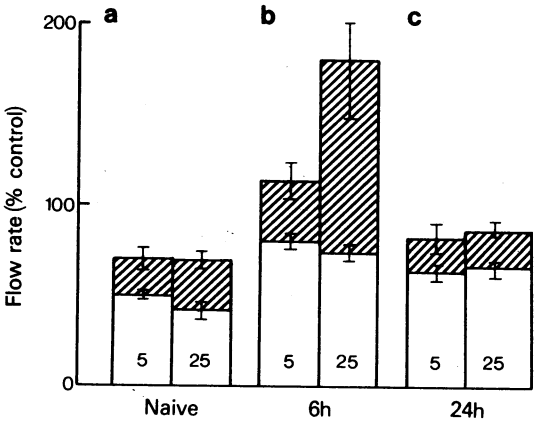

Figure 5 The effects of morphine (5 and 25 mg/kg) before (open columns) and after naloxone (40 mg/kg, hatched columns) on nerve-induced secretion by the parotid gland during stimulation at 2.5 Hz. (a) Control animals (n = 9); (b) morphine-pretreated animals, 6 h after pellets were removed (n = 8); (c) morphine-pretreated animals, 24 h after pellets were removed (n = 7). The secretion is expressed as a percentage of control flow rate (ordinate scale) before the drugs were administered; the dose of morphine (mg/kg) is shown within each column; s.e. means are indicated by vertical lines.

Tolerance to morphine, as measured by its effect on pulmonary minute volume, appeared to be well developed by the 3rd day of implantation. The ventilatory depression produced by 5 and 20 mg/kg morphine when measured on the 3rd, 6th and 10th days was the same; there was no significant difference between the 3 groups. Throughout a 10 day period, approximately 20 mg of morphine base was released from the pellets per day (Sowell, Bowen & Carpenter, 1977). This resulted in a four fold increase in morphine tolerance, which remained constant after the 3rd day.

Parotid secretion in morphine pretreated rats

Goldstein & Schultz (1973), and Johnson, Westfall, Fleming & Howard (1977) found that tolerance in autonomic structures may not develop as rapidly as it does in the CNS. A summary of the effects of 5 and 25 mg/kg morphine on nerve-induced (2.5 Hz) flow rate in control and in morphine-pretreated animals is shown in Figure 5. Pellets were implanted for six days, after which they were removed for either 6 or 24 h before the experiment was begun. There was less depression of secretion by morphine in both groups of pretreated animals; in control animals, morphine (5 and 25 mg/kg) diminished salivary flow rate by 50 and 58% respectively (Figure 5 1st and 2nd columns), whereas these same doses reduced the flow rate by only 20 and 27% respectively in the morphine-pretreated animals (Figure 5 3rd and 4th columns) from which the pellets had been removed for 6 h. In those animals represented by the 5th and 6th columns of Figure 5, pellets were excised 24 h before the experiment. A 5 mg/kg dose of morphine under these conditions reduced salivary flow by 37%, which was a greater reduction than shown in the 6 h group. An analysis of variance showed that flow rates after 5 mg/kg morphine were different for the control, 6 and 24 h groups. The depressant action of a 25 mg/kg dose of morphine was always greater in control than in morphine pretreated animals, yet the effect of this dose in the 6 or 24 h group did not differ significantly from the 5 mg/kg dose. Although the effects of the two doses were different in the control animals the difference became obscure with the development of tolerance.

The depression of nerve-induced secretion by mor-

phine was reversed by naloxone (40 mg/kg) as shown by the hatched columns in Figures 2 and 5. The reversal was incomplete in control animals and in the morphine-pretreated animals 24 h after pellet removal (Figure 5, columns 5 and 6). However, after naloxone, nerve-induced secretory responses were greater than control 6 h after pellet removal (Figure 5, columns 3 and 4). After the implantation of morphine pellets, the parotid gland developed a tolerance to the depressant effects of morphine which was manifest 6 and 24 h after the pellets were removed. However, 6 h after pellet removal the parotid gland became hyper-responsive after naloxone; when the latter animals were treated with naloxone after morphine (25 mg/kg), nerve-induced secretion was increased by 140%. This enhancement of secretion was not observed 24 h after pellet removal: secretion in morphine-pretreated animals 24 h after pellet removal and after naloxone was not different from control animals (Figure 5, columns 1 and 2).

Cation content of parotid saliva

Sodium and potassium are the principal cations in rat parotid saliva, with calcium being only a minor component. When salivary flow rate is maximal (40 to 50 µl/min), Na^+ content of saliva is high (140 to 160 mEq/l), decreasing to 60 to 80 mEq/l at low rates of flow (Woodruff & Carpenter, 1973). In the present series of experiments, the salivary flow produced in 6 control rats during stimulation at 2.5 Hz was 9.6 ± 1 µl/min, with a Na^+ content of 89 ± 5 mEq/l.The effects of morphine (25 mg/kg) given to these same animals on major cationic content of saliva are summarized in Table 1. In our present study, a dependent relation between Na^+ content and flow rate could not be established unequivocally; the reduction by over 50% in salivary flow produced by morphine did not result in a parallel reduction in Na^+ content.

Discussion

Morphine affects autonomic neuro-effector transmission in a highly selective manner with respect to stimulus frequency and site of action. Morphine inhibits the release of ACh from nerves in the guinea-pig ileum (Paton, 1957) and vagal slowing of the rabbit heart (Kosterlitz & Taylor, 1959) but has no inhibitory effect on cholinergic transmission in the guinea-pig heart (Kosterlitz & Taylor, 1959) or the rabbit ileum (Greenberg, Kosterlitz & Waterfield, 1970). In at least 2 other organs, namely the mouse vas deferens (Henderson, Hughes & Kosterlitz, 1974) and the cat nictitating membrane (Cairnie *et al.*, 1961), junctional transmission is depressed by morphine but also only at low stimulus frequencies. At cholinergic junctions which are susceptible to morphine there is a greater release of acetylcholine with each stimulating pulse at low frequencies (Paton, 1963). This relation between stimulus rate and transmitter output is not found at autonomic junctions that are insensitive to morphine (Henderson *et al.*, 1974).

The sensitivity of muscarinic receptors in the parotid gland was not diminished by morphine; in control and morphine-pretreated animals the secretion produced by the parotid gland during ACh infusion was not depressed. Accordingly, the depression of nerve-induced secretion by morphine as seen in our study is likely to be prejunctional. On the other hand, the enhanced flow rate during ACh infusion after morphine may be related to an inhibition of cholinesterase (Johanesson, 1962); during the infusion more ACh may reach the gland in the treated rats since it would undergo less hydrolysis by plasma cholinesterases.

A dose of 5 mg/kg was lethal in 7 out of 16 control rats anaesthetized with Dial-urethane: pulmonary minute ventilation in the surviving animals was reduced to 50% of control. However, morphine-pretreated animals tolerated 20 mg/kg doses of morphine and there was no ventilatory arrest by this dose for up to 24 h after the pellets were removed. Morphine also produced less depression of nerve-induced secretion in morphine-pretreated animals. This tolerance was less after the pellets had been removed for more than 24 h.

The action of naloxone 6 h and 24 h after the pellets were removed was remarkably different. In control

Table 1 Effect of morphine on nerve-induced (2.5 Hz) salivary flow rate and cation content

	Control	*Morphine-treated (25 mg/kg)*
Flow rate (µl/min)	9.6 ± 1	4.2 ± 0.9*
Na^+ (mEq/l)	89.0 ± 5	68.8 ± 12 (NS)
K^+ (mEq/l)	16.5 ± 0.4	18.5 ± 0.57*
Ca^{2+} (mEq/l)	6.0 ± 0.21	5.7 ± 0.13 (NS)

Values are mean ±s.e. mean; $n = 6$.
*Significantly different from control ($P < 0.05$); NS: not significant.

animals the depressant effect of morphine (25 mg/kg) on nerve-induced secretion at 2.5 Hz was antagonized by naloxone. However, in pretreated animals given 25 mg/kg morphine 6 h after removal of the pellets, the secretion of the parotid gland during nerve stimulation at 2.5 Hz was more than doubled after naloxone. This action of naloxone was not as great after 5 mg/kg morphine. When the pellets had been removed for 24 h, the administration of naloxone to morphine-pretreated rats produced secretory flow rates during stimulation that were the same as control animals.

The difference between the 6 h and 24 h groups is probably due to the extent of the 'withdrawal' precipitated by naloxone. After 24 h the animals displayed many typical signs of withdrawal such as diarrhoea, 'wet dog' shakes and weight loss amounting to 10% of the body weight. After 6 h these signs were not evident; the enhancement of secretory flow by naloxone occurred before 'withdrawal'. Precipitated withdrawal would be less intense 24 h after the pellets had been removed.

Tolerance and withdrawal may be the consequence of a suppression of transmitter release in the CNS by the narcotic. When administered for extended periods, transmitter release becomes tolerant to the narcotic but, when it is suddenly discontinued (as during precipitated withdrawal), excessive amounts of transmitter may be released, resulting in hyperactivity. Our findings with the rat parotid gland are consistent with this proposal.

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(Received September 5, 1977. Revised July 19, 1978.)